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Differential expression of calcium/calmodulin-regulated *SlSRs* in response to abiotic and biotic stresses in tomato fruit

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Calcium has been shown to enhance stress tolerance, maintain firmness and reduce decay in fruits. Previously we reported that seven tomato *SlSRs* encode calcium/calmodulin-regulated proteins, and that their expressions are developmentally regulated during fruit development and ripening, and are also responsive to ethylene. To study their expressions in response to stresses encountered during postharvest handling, tomato fruit at the mature-green stage was subjected to chilling and wounding injuries, infected with *Botrytis cinerea* and treated with salicylic acid or methyl jasmonate. Gene expression studies revealed that the seven *SlSRs* differentially respond to different stress signals. *SlSR2* was the only gene upregulated by all the treatments. *SlSR4* acted as a late pathogen-induced gene; it was upregulated by salicylic acid and methyl jasmonate, but downregulated by cold treatment. *SlSR3L* was cold- and wound-responsive and was also induced by salicylic acid. *SlSR1* and *SlSR1L* were repressed by cold, wounding and pathogen infection, but were upregulated by salicylic acid and methyl jasmonate. Overall, results of these expression studies indicate that individual *SlSRs* have distinct roles in responses to the specific stress signals, and *SlSRs* may act as a coordinator(s) connecting calcium-mediated signaling with other stress signal transduction pathways during fruit ripening and storage.

Introduction

Fleshy fruits are perishable and their quality is impacted by both abiotic and biotic stresses. About 25–40% of all fresh produce worldwide is lost after harvest (Hodges et al. 2011). The postharvest losses for fresh produce are mainly caused by chilling and mechanical injuries, and decay due to fungi and bacteria during handling, storage and transportation. Unlike dry seeds and fruits, fleshy fruits can cope with these abiotic and biotic stresses by modulating the expression of genes involved in ethylene, salicylic acid (SA), jasmonic acid (JA) or its potent volatile form methyl jasmonate (MeJA) and other signaling pathways (Ding et al. 2002, Giovannoni 2004, Wasternack 2007, Rohwer and Erwin 2008, Yu et al. 2009, Asghari and Aghdam 2010, Robert-Seilaniantz et al. 2011).

Calcium is most frequently associated with stress tolerance, fruit firmness, ripening and senescence (Shear 1975, Poovaiah 1986, Martin-Diana et al. 2007). Pre- and Postharvest calcium treatments maintain fruit firmness and prevent decay in both climacteric and nonclimacteric fruits (Poovaiah and Shekhar 1978, Conway and Sams 1987, Abbott et al. 1989, Sams et al. 1993, Saftner et al. 1999, Saftner et al. 2003, Park et al. 2005, Ritenour et al. 2006). Calcium is directly

Abbreviations – JA, jasmonic acid; MeJA, methyl jasmonate; qPCR, quantitative real-time PCR; SA, salicylic acid.

and integrally involved in texture changes and in maintaining fruit quality (Raese and Drake 1993, Raese and Drake 2006). However, the molecular mechanisms of calcium-mediated signaling in stress tolerance during fruit ripening and storage are still not clear.

Accumulating evidence indicates that calcium is a universal second messenger involved in sensing and recognition of a variety of abiotic and biotic signals such as temperature, wounding and pathogen attack, and mediates plant responses to these stress signals (Poovaiah et al. 1987, Poovaiah and Reddy 1993, Trewavas and Malho 1998, Reddy 2001, Sanders et al. 2002, Xiong et al. 2002). Cellular calcium changes can be sensed and interpreted by calcium-binding proteins that function as signal sensors. Calmodulin is one of the most well characterized calcium-sensors and functions as a modulator of other target proteins (Snedden and Fromm 2001, Reddy et al. 2002, Yang and Poovaiah 2003, Bouche et al. 2005, Yamaguchi et al. 2005). In recent years, many calcium and calcium/calmodulin binding transcription factors have been identified in plants (Reddy et al. 2011). The SR/CAMTA transcription factor family is important in the plant response to multiple abiotic and biotic stresses, including cold, wounding, drought and pathogens, as well as stress-related hormonal signals like ethylene, auxin, MeJA and SA (Reddy et al. 2000, Yang and Poovaiah 2000, Bouche et al. 2002, Yang and Poovaiah 2002, Galon et al. 2010). The knockout of a *SR/CAMTA* (*AtSR1*) in *Arabidopsis* led to increased accumulation of SA and enhanced disease resistance to both *Pseudomonas syringae* and *Botrytis cinerea* (Galon et al. 2008, Du et al. 2009). In contrast, *AtSR1* is a negative regulator for JA biosynthesis and herbivory tolerance (Laluk et al. 2012, Qiu et al. 2012). A double knockout mutant (*AtSR1* and *AtSR2*) exhibited reduced tolerance to low temperature (Doherty et al. 2009). The genes affected by *AtSR1* include *PR* genes, expansin, β-1,3-glucanase, phospholipase A2, accelerated cell-death protein 6 and senescence associated gene 21 (Galon et al. 2008). The *SR/CAMTA*s' primary target of CGCG-box has been suggested to be the major calcium-regulated *cis*-element (Kaplan et al. 2006) and the rapid wounding responsive element (Walley et al. 2007). In an effort to investigate the functions of *SR/CAMTAs* during fruit ripening and storage, we cloned all seven *SR/CAMTA* orthologs in the model horticultural crop tomato (*Solanum lycopersicum*) and characterized their differential expression patterns during fruit development and ripening (Yang et al. 2012). All seven *SlSR*s were dramatically altered in ripening mutant *rin* (ripening-inhibitor) compared with wild type fruit. Moreover, ethylene treatment of fruit at the mature-green stage transiently stimulated expression of all *SlSR*s. Therefore, we hypothesize that *SlSRs* are an important player for regulating fruit ripening downstream of *RIN*. Here we report the gene expression patterns of *SlSR*s in tomato fruit in response to low temperature and mechanical injury, and the necrotrophic fungal pathogen *B. cinerea*, as well as treatments with the signal molecules SA and MeJA.

Materials and methods

Plant materials

Tomato plants (*S. lycopersicum* cv. Moneymaker) were grown in a greenhouse at 28°C with a 16/8 h (light/dark) cycle. Fruits were harvested at the mature green stage (MG), as defined by USDA-ARS criteria (http://www.ba.ars.usda.gov/hb66/138tomato.pdf), when physiologically mature but not yet ripening. Specifically, the fruit surface is completely green, the shade varying from light to dark. After cutting the fruit, seeds become tan (mature seeds), and gel formation is observed in at least two locules or all locules have gel and internal color is still green. In the industry, tomatoes are often harvested at this stage for packing and shipment, and subsequently treated with ethylene to promote ripening prior to sale. The greenhouse-grown MG fruits were held under ambient conditions overnight to reduce harvest shock prior to treatment.

Cold, MeJA and SA treatments

Cold treatment was applied by immersing fruits in cold water (4°C) for 0–4 h. For SA treatment, fruits were immersed in solutions of 0, 1, 4, or 16 m*M* SA for 8 h, or were treated with 4 m*M* SA for 0–48 h. For MeJA treatment, fruits were sealed in a jar with 20 µ*M* MeJA vapor. After each treatment, pericarp samples were immediately frozen in liquid nitrogen and stored at −80°C.

Wounding treatment and pathogen infection

Fruits were mechanically injured and infected by inoculation with spores of the fungal pathogen *Botrytis cinerea* as described with modification (Cantu et al. 2009). *B. cinerea* was isolated from decayed raspberry fruit harvested from field plots maintained at the Beltsville Agricultural Research Center located in Beltsville, MD. Conidia were collected from 7-days-old sporulating cultures and suspended in deionized water. Conidia in the suspension were quantified using a hemacytometer and the titer was adjusted to 1×10^4 conidia ml^{-1}. Fruits were punctured (3 mm

depth, 2 mm diameter) at six sites around the equator of each fruit; three sites with 10 µl of conidial suspension, and the three with 10 µl of sterile Tween20-treated water. Healthy fruit remained intact and did not receive any treatment. After inoculation, the fruits were stored in plastic sealed containers with moist towels to maintain high humidity and kept at 20°C. Pericarp tissue samples were obtained from inoculated and wounded fruit by using a cork borer to isolate the tissue immediately surrounding the inoculated area at different intervals of time after treatment. The pericarp tissue collected from fruit at the different time points (0, 1, 2, 4, 24 and 48 h) was frozen in liquid nitrogen and stored at −80°C.

RNA preparation and RT-qPCR analysis

Total RNA was isolated from frozen tissue using the RNeasy Plant Mini Kit following the manufacturer's instructions (Qiagen, Valencia, CA). After DNase digestion, the absorbance at 260 nm was measured using a nanodrop spectrophotometer to ensure an equal amount of RNA from each tissue sample was used in the cDNA synthesis reactions. One microgram of total RNA was used to synthesize cDNA with the oligo-$(dT)_{18}$ primer following the instructions of the Superscript III kit (Invitrogen). Quantitative real-time PCR (qPCR) analysis of cDNA was performed on a CFX96 Real-Time System (Bio-RAD, Hercules, CA) with *SlSR* gene-specific primers (Yang et al. 2012). Other gene-specific primers used were: *SlCBF1* (AY034473), GTGTGGAAACTGA TGCCTAC /ATGTTCATGTATCCCGGCCA; *SlPR1* (NM_001247429), CTGTGAAGATGTGGGTTGATGA G/TCTCCAGTTACCTGGTGGATCAT and *SlPR2b* (M80608), TCTTGCCCCATTTCAAGTTC/TGCACGT GTATCCCTCAAAA. The efficiency coefficient E was calculated for all primer pairs individually by plotting the relationship between Ct value (threshold cycle) and log[cDNA]. The following thermal cycle conditions were used: 95°C for 2 min, followed by 45 cycles of 95°C for 5 s and 60°C for 20 s. All reactions were performed in triplicate from three independent samples. Following PCR, a melting curve analysis was performed. Relative quantification of specific mRNA levels was analyzed using the cycle threshold (Ct) $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). It has been reported that actin may not be the best reference gene in tomato leaves during the stress treatment (Løvdal and Lillo 2009). Thus we examined nine housekeeping gene expressions in fruit under various stresses. Those genes included actin, β-tubulin, elongation factor 1α, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, ribosomal protein L2, ubiquitin and a catalytic subunit of protein phosphatase 2A. It turned out that actin in fruit had the best stability in all treatments. Thus relative expression levels were normalized using actin (accession number: X55749) as the reference gene and shown in percentage (highest value = 100%). Student's *t*-test ($P_{0.05}$) was used to determine the significant difference of relative expression of individual genes among nontreatment and different treatments (Microsoft Excel 2007).

Results

Response of *SlSRs* to low temperature

Tomato is a subtropical crop, and tomato fruit are susceptible to chilling injury (Ding et al. 2002). Previous studies show that cold treatment stimulates the expression of most *SA/CAMTAs* in *Arabidopsis* seedlings (Yang and Poovaiah 2002). To study the effects of low temperature exposure on expression of *SlSRs* in tomato fruit, mature green tomatoes were chilled at 4°C for 0–4 h. Seven *SlSRs* showed a differential response to cold stress (Fig. 1). Cold treatment reduced the expression of four of the seven *SlSRs*, including *SlSR1*, *SlSR1L*, *SlSR3* and *SlSR4*. The maximal reduction of *SlSR1* and *SlSR4* were 6.8- and 4-fold, respectively, whereas the inhibitory effects on *SlSR1L* and *SlSR3* expression were relatively weak (approximately twofold). In contrast, cold treatment stimulated the expression of *SlSR2*, *SlSR2L* and *SlSR3L*. The most dramatic cold stimulatory effect was observed for *SlSR2*; its expression was scarcely detected in nontreated fruit but was quickly induced at chilling temperature. *SlSR2* transcript levels reached a maximum after 1 h of cold treatment and gradually declined thereafter. In comparison, the cold stimulation of *SlSR2L* and *SlSR3L* expression was relatively low (less than approximately 30%). *SlCBF1*, a well defined cold-responsive gene (Zhang et al. 2004), showed cold-induced expression as expected. These results indicate that most of the *SlSRs* are early cold responsive genes, and that *SlSR2* is a major cold-upregulated gene, whereas *SlSR1* is a major cold repressed gene.

Responses to mechanical injury and pathogen infection

Wounding or mechanical injury of tomatoes is another common type of damage occurring during postharvest handling. Wounding not only has a negative impact on fruit quality, but also facilitates infection and decay by postharvest pathogens. Therefore, we studied the effects of both wounding and pathogen infection on expression of *SlSRs*. Tomato fruits were injured by puncturing holes in the outer pericarp and then only injected with water or inoculated with an aqueous

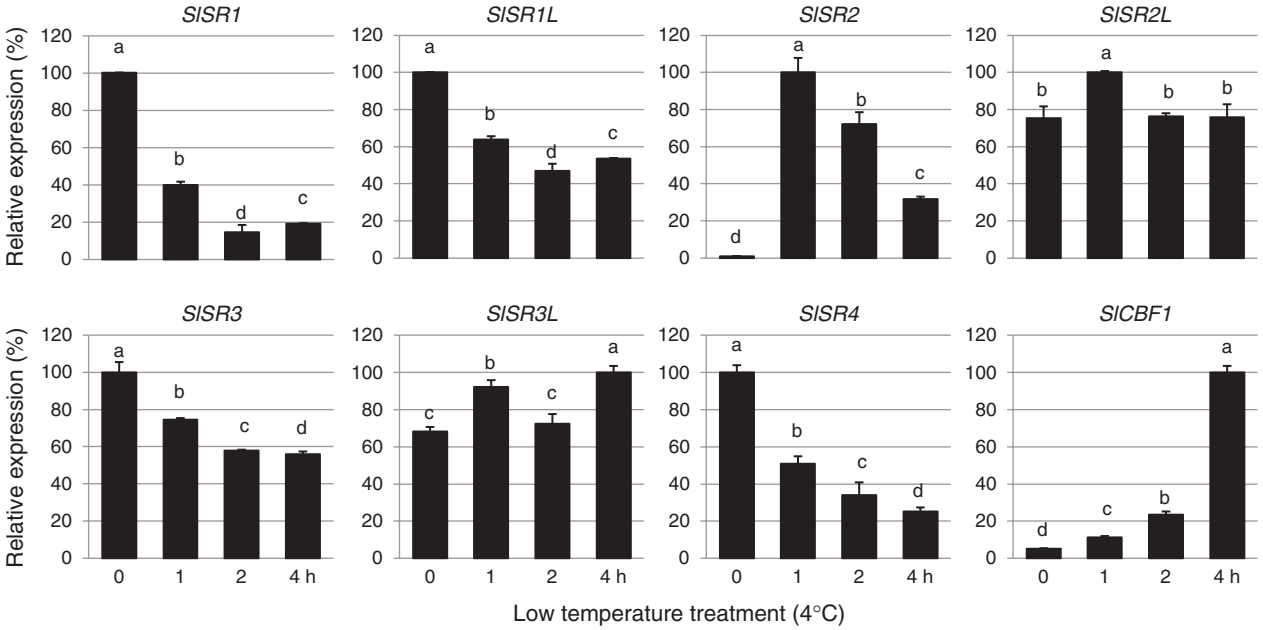

Fig. 1. Expression of *SlSRs* in tomato fruit held at low temperature. Tomato fruit at the mature-green stage were chilled at 4°C for 0, 1, 2 or 4 h. Total RNA samples for RT-PCR were isolated from pericarp tissue. Transcript levels of *SlSR* genes were investigated by RT-qPCR. Relative gene expression levels (highest value = 100%) are shown following normalization with actin transcript values. Bars represent the range given for *SlSRs* mRNA abundance relative to untreated control samples (0 h). For each gene, different letters indicate statistically significant differences among mean values (P-value < 0.05; t-test). The qPCR analyses were repeated at least three times from two independent experiments with similar results.

suspension of conidia of *B. cinerea* (Fig. 2A). The growth of the fungus at 1 day postinoculation (1 dpi) was not evident and soft rot symptoms did not develop. Water soaking and slight necrosis were localized at the sites of inoculation as early as 1 dpi but were not evident in the wounded, uninoculated sites. At 2 dpi, water soaking and necrosis were more pronounced around the inoculation sites as the pathogen began to grow outward in a radial pattern. After 3 dpi, the fruit infected with *Botrytis* displayed extensive necrotic decay, including tissue softening and grayish colored mycelium at the lesion site, whereas the wounded fruit had begun to show slight water soaking and minor necrosis at the inoculation point (data not shown).

The expression levels of *SlSRs* in response to wounding and pathogen treatments were quantified. To confirm that the treatments were appropriate, we examined the expression patterns of *SlPR1* and *SlPR2b*, two genes induced mainly by pathogens and SA, but also by wounding, JA, etc. (Robert-Seilaniantz et al. 2011). *SlPR1*'s function is still unknown and *SlPR2b* is a β-glucanase gene involved in degradation of the fungal pathogen cell wall. As shown in Fig. 2B, both *SlPR1* and *SlPR2b* were induced by wounding and pathogen inoculation. For *SlPR1*, the obvious stimulation was observed at 24 h when necrotic symptoms began, and the highest expression appeared at 48 h after inoculation when necrotic symptoms of the pathogen were clearly evident. However, the expression level in wounded fruit was only about 7% of that observed in pathogen inoculated fruit. For *SlPR2b*, in addition to a major peak at 48 h for both wounding and pathogen inoculation, there was a small peak (about 2% of the highest expression level) at 2 h after wounding. These results suggest that the first peak is the rapid response to wounding and the second peak is a late response to wounding and/or the pathogen.

Wounding and pathogen inoculation showed similar effects on expression of *SlSRs* in most cases (Fig. 2B). Responses can be categorized as four types of expression patterns. First, both wounding and infection downregulated *SlSR1*, *SlSR1L* and *SlSR3* expression. The maximal inhibition for *SlSR1* and *SlSR1L* (approximately three- to sixfold for wounding and approximately 2.5-fold for the pathogen) appeared 48 h after treatment. *SlSR2L* decreased expression (about four- to fivefold) within 2–4 h after treatment, then slowly increased to 60–80% of maximal expression. Second, both wounding and infection upregulated *SlSR2* and *SlSR3L* expression. *SlSR2* showed two peaks; one small transient peak appeared 1 h after treatment (about 18–19% of the

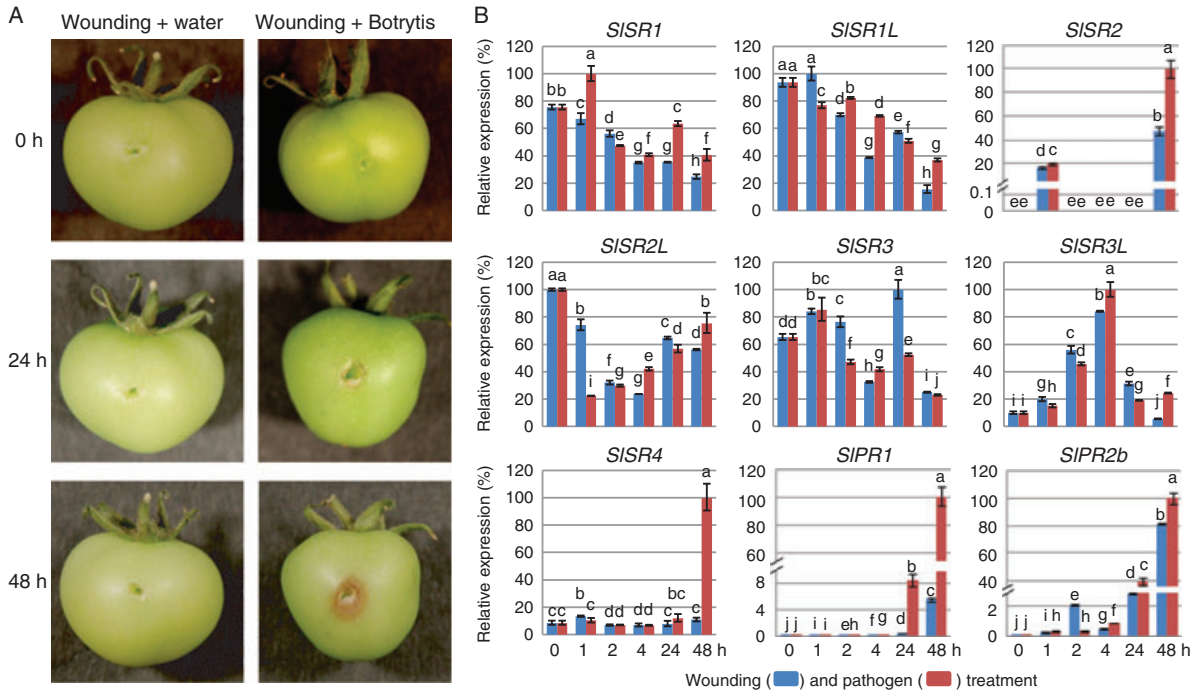

Fig. 2. Pathogen infection and wounding effects on expression of *SlSRs*. Tomato fruit at the mature-green stage were mechanically injured and the wound immediately treated with water or inoculated with conidia of *Botrytis cinerea*. The wounded and wounded plus infected areas were excised after 0–48 h of incubation at 20°C. (A) Wounded and *B. cinerea* inoculated fruit at 0, 24 and 48 h showing the progression of *B. cinerea* decay. (B) *SlSR* expression patterns in response to wounding and *B. cinerea*. Total RNA samples for RT-PCR were isolated from pericarp tissue and transcript levels of *SlSR* genes were determined by RT-qPCR. Relative gene expression levels (highest value = 100%) are shown following normalization with actin transcript values. Bars represent the range given for *SlSRs* mRNA abundance relative to untreated control samples (0 h). For each gene, different letters indicate statistically significant differences among mean values (P-value < 0.05; t-test). The qPCR analyses were repeated at least three times from two independent experiments with similar results.

maximal expression) followed by a second major peak at 48 h, which was over twofold greater in *Botrytis* infected compared with wounded fruit. By contrast, *SlSR3L* showed a bell-shaped distribution of expression in response to wounding and pathogen infection with a peak 4 h after treatment. Third, *SlSR4* induction was only observed in fruit 48 h after pathogen treatment, suggesting that *SlSR4* is a late pathogen upregulated gene. Fourth, *SlSR3* showed irregular expression patterns in response to both wounding and pathogen infection.

Response to SA treatment

To study the effect of SA on expression of *SlSRs*, fruits were treated with different concentrations of SA ranging from 0 to 16 m*M* for 8 h (Fig. 3). Expression of all seven *SlSRs* was SA-upregulated. *SlSR1*, *SlSR1L* and *SlSR3* showed a similar dosage-dependent expression pattern with the maximum transcript level induced by 16 m*M* SA treatment. SA increased their expression by about six-, five- and fourfold, respectively. In comparison, *SlSR2*, *SlSR2L*, *SlSR3L* and *SlSR4* had the stimulatory peak at 4 m*M*. The expression of *SlSR2L*, *SlSR3L* and *SlSR4* was increased by three-, two- and fourfold, respectively. *SlPR1*, the defined SA-responsive gene, showed the highest SA induction of expression at 4 m*M*.

Further, we selected 4 m*M* SA to treat fruit for different time periods ranging from 0 to 48 h (Fig. 4). As in the prior experiment, all the *SlSRs* were upregulated by SA, and the stimulatory effect was observed as early as 2 h after treatment. However, the obvious stimulatory effect for all the *SlSRs* except *SlSR2* and *SlSR4* was observed after 8 h of SA treatment. *SlSR2* was scarcely detected in untreated fruit but was markedly induced by SA. As compared with the untreated control, the expression of *SlSR1*, *SlSR1L* and *SlSR3L* was increased by 13-, 6- and 6-fold, respectively. SA stimulatory effects on expression of *SlSR2L*, *SlSR3* and *SlSR4* were less pronounced, only about 2.5-fold relative to the untreated controls. Because the expression patterns of *SlSRs* after SA treatment were similar to that of *SlPR1*, a SA-late responsive marker gene, this suggests that all *SlSRs* are late SA-responsive genes (Uknes et al. 1993, Uquillas et al. 2004).

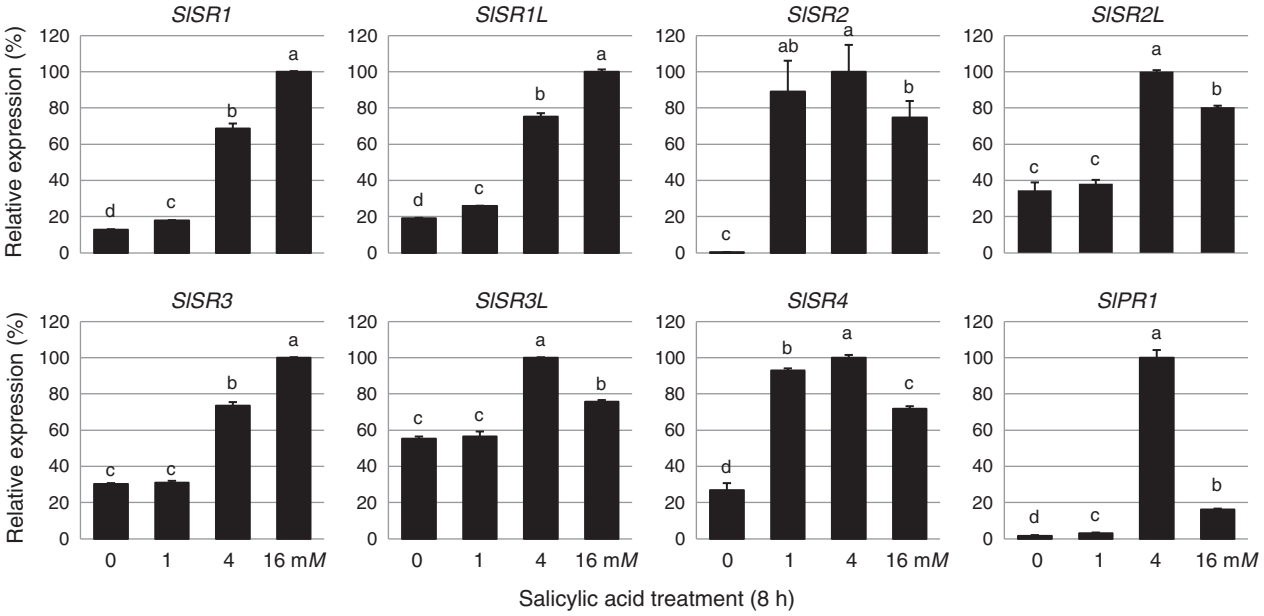

Fig. 3. Salicylic acid dosage effect on expression of *SlSRs*. Tomato fruit at the mature-green stage were treated with 0, 1, 4, 16 m*M* SA for 8 h. Total RNA samples used for RT-PCR were isolated from pericarp tissue. Transcript levels of *SlSR* genes were determined by RT-qPCR. Relative gene expression levels (highest value = 100%) are shown following normalization with actin transcript values. Bars represent the range given for *SlSRs* mRNA abundance relative to untreated samples (0 h). For each gene, different letters indicate statistically significant differences among mean values (*P*-value < 0.05; *t*-test). The qPCR analyses were repeated at least three times from two independent experiments with similar results.

Response to MeJA treatment

The temporal expression patterns of *SlSRs* to JA were studied after treating fruit with 20 µ*M* MeJA, a potent volatile form of JA, for different time periods ranging from 0 to 48 h. All *SlSR* expression levels were increased by MeJA except *SlSR3L*, which showed decreased expression in the first 4 h and then returned to the basal level after 8 h (Fig. 5). The greatest stimulation for *SlSR2* and *SlSR4* appeared at 8 and 4 h, respectively, whereas the expression for *SlSR1*, *SlSR1L*, *SlSR2L* and *SlSR3* peaked at 24 h. The MeJA stimulatory effect for *SlSR1*, *SlSR1L*, *SlSR2L* and *SlSR3* was about a four- to fivefold increase. However, *SlSR2* exhibited the most dramatic response to MeJA treatment. *SlSR2* expression was not detected at times 0 and 2 h but was clearly evident at 4 h and peaked at 8 h. As expected, the control gene *SlPR2b* was induced by MeJA and reached the highest expression level after 8 h treatment. These results suggest that all the *SlSRs* except *SlSR3L* are MeJA-upregulated late-responsive genes.

Discussion

Chilling injury and mechanical injury are two major abiotic stresses during storage of harvested fruits, especially for tropical and subtropical fruits. These two injuries also greatly increase susceptibility of the fruit to decay resulting from broad host range necrotrophic pathogens such as *B. cinerea*. It is documented that these abiotic and biotic stresses can trigger the biosynthesis of several signaling molecules, such as SA (Ogawa et al. 2010) and JA (Wasternack 2007), which have been shown to be important components of microbial pathogen defense pathways. MeJA has been used in postharvest applications to reduce the development of chilling injury symptoms in a number of horticultural crops, including loquat, tomato and peach fruit (Ding et al. 2002), and to activate of plant defense mechanisms (Rohwer and Erwin 2006). SA is a key signaling molecule for the activation of defense genes in response to both biotic and abiotic stresses (Ding et al. 2002, Asghari and Aghdam 2010, Robert-Seilaniantz et al. 2011). SA treatment of harvested fruits can help to reduce decay incidence and alleviate chilling injury by activating defense genes such as *PR-1* and *PR-2* (Tornero et al. 1997, Uquillas et al. 2004, Rohwer and Erwin 2006, van Loon et al. 2006, Asghari and Aghdam 2010). Here we show that SA can stimulate gene expression of all *SlSRs*, and MeJA can also trigger *SlSR* expression with the exception of *SlSR3L*, suggesting that *SlSRs* are generally SA and MeJA-upregulated genes (summarized in Table 1). However,

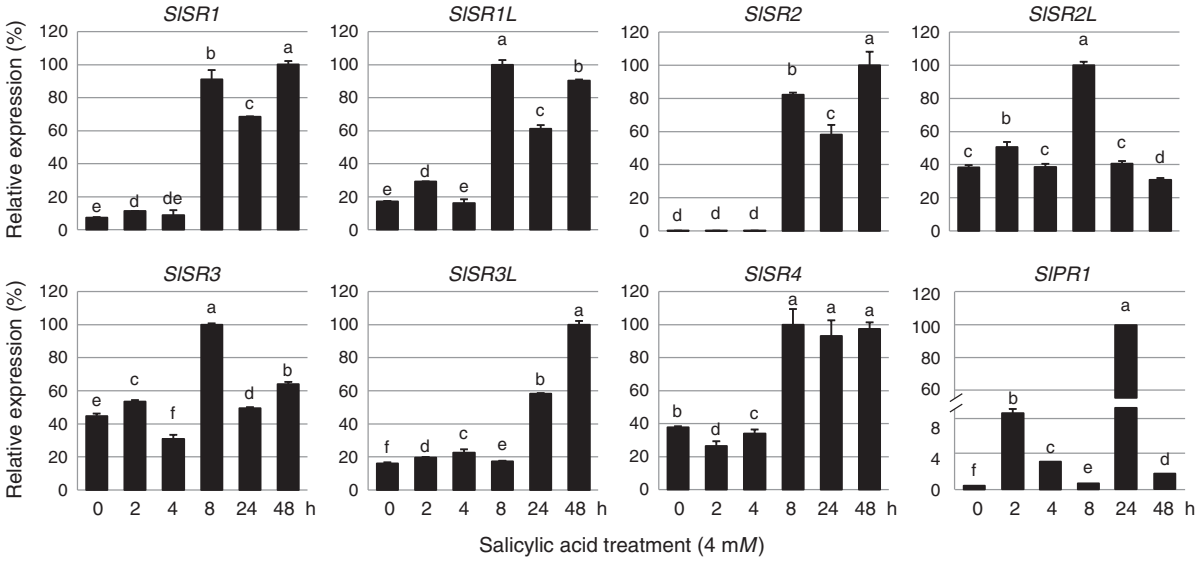

Fig. 4. Time course of salicylic acid effects on expression of *SlSRs*. Tomato fruit at the mature-green stage were treated with 4 m*M* SA for 0, 2, 4, 8, 24 and 48 h. Total RNA samples used for RT-PCR were isolated from pericarp tissue. Transcript levels of *SlSR* genes were determined by RT-qPCR. Relative gene expression levels (highest value = 100%) are shown following normalization with actin transcript values. Bars represent the range given for *SlSRs* mRNA abundance relative to untreated samples (0 h). For each gene, different letters indicate statistically significant differences among mean values (*P*-value < 0.05; *t*-test). The qPCR analyses were repeated at least three times from two independent experiments with similar results.

chilling temperature, mechanical injuries and pathogen infection did not induce upregulation of all members of the *SlSR* gene family. Only *SlSR2* exhibited an increase in transcript levels in response to all the stresses. To the contrary, *SlSR1* and *SlSR1L* were found to be cold, wounding and pathogen repressed genes, *SlSR4* was repressed by cold yet upregulated by pathogen infection, and *SlSR2L* was downregulated in response to both wounding and the fungal pathogen *B. cinerea*. Therefore, their expression may be subjected by more complex control mechanisms. Note that basically all *SlSRs* are late SA and MeJA responsive genes because their upregulation was clearly evident after 8–24 h of treatment (Figs. 4 and 5). Late responsive genes are those genes such as *PR1* gene that are activated after several hours of treatment through a process dependent on de novo protein synthesis (Uknes et al. 1993). By contrast, *SlSRs* are induced or repressed by cold and wounding treatments within 1 hour, suggesting they are early responsive genes to abiotic stresses, but late responsive genes to biotic stresses.

Like most plant genes, *SlSRs* belong to a small gene family. Seven members of this family share a structural similarity with a DNA-binding domain in the N-terminus, calcium/calmodulin-binding domain in the C-terminus, and ankyrin repeats in the central region (Bouche et al. 2002, Yang and Poovaiah 2002). Although we cannot exclude gene redundancy, individual *SlSRs* appear to have specific functions based on their temporal and spatial gene expression patterns and responses to different signals. For example, *SlSR4* had a strong positive induction response to pathogen infection, while *SlSR1*and *SlSR1L* were significantly repressed by cold and wounding. In particular, *SlSR2* is a unique gene in that it is responsive to multiple signals. During fruit development, *SlSR2* expression is suppressed at MG and breaker stages but it is highly expressed in MG-equivalent stage fruit of *rin*, a ripening inhibited mutant. In all tested stress treatments, *SlSR2* showed a significantly positive response. Further functional studies are needed to determine the importance of individual *SlSR* genes in postharvest stress responses.

Plant responses to environmental and hormonal cues often involve calcium as a second messenger (Poovaiah et al. 1987, Poovaiah and Reddy 1993, Trewavas and Malho 1998, Reddy 2001, Sanders et al. 2002, Xiong et al. 2002). Calcium functions through binding to calcium sensors such as calmodulin, which regulates calcium/calmodulin-target proteins, thereby leading to physiological responses (Snedden and Fromm 2001, Reddy et al. 2002, Yang and Poovaiah 2003, Bouche et al. 2005, Yamaguchi et al. 2005). *SlSRs* belong to the SR/CAMTA family, which comprises an array of calcium/calmodulin-binding transcription

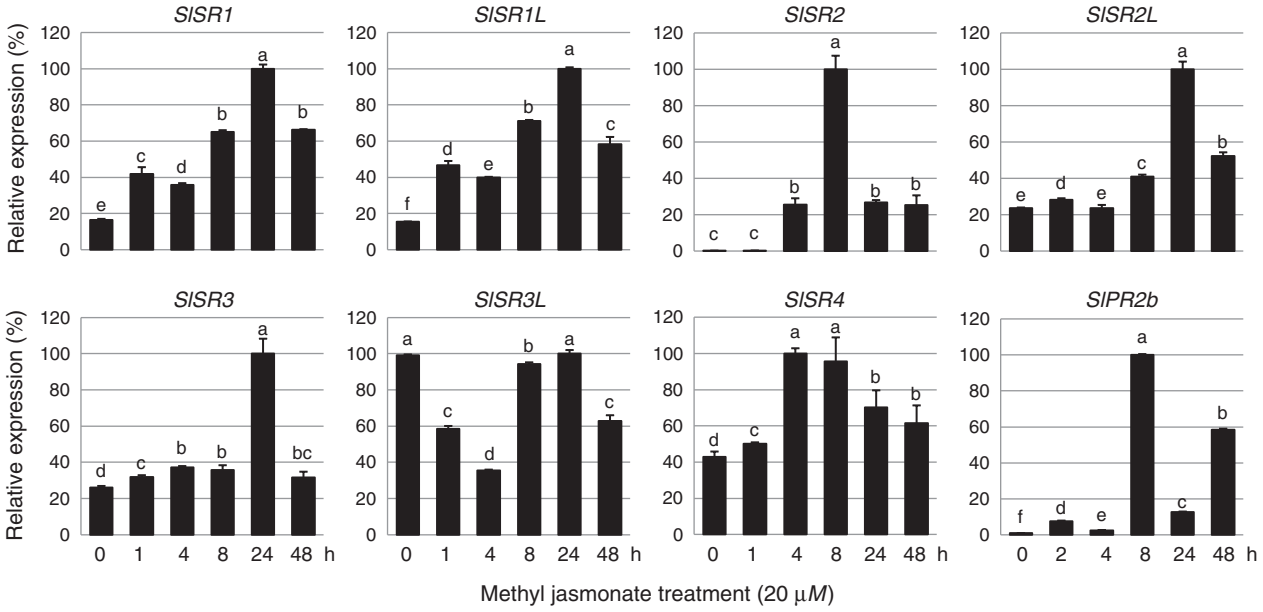

Fig. 5. Time course of MeJA effects on expression of *SlSR*s. Tomato fruit at the mature green stage were treated with 20 μ*M* MeJA for 0, 2, 4, 8, 24 and 48 h. Total RNA samples used for RT-PCR were isolated from pericarp tissue. Transcript levels of *SlSR* genes were investigated by RT-qPCR. Relative gene expression levels (highest value = 100%) are shown following normalization with actin transcript values. Bars represent the range given for *SlSRs* mRNA amount relative to untreated control samples (0 h). For each gene, different letters indicate statistically significant differences among mean values (*P*-value < 0.05; *t*-test). The qPCR analyses were repeated at least three times from two independent experiments with similar results.

Table 1. *SlSRs* expression in response to different stress signals. Fold changes of individual gene expression levels after each treatment are indicated. The fold changes in expression are based on the highest or lowest relative expression level of each gene during the entire time course after a specific treatment in comparison with its relative expression level in nontreated fruit at 0 h. +, upregulation; −, downregulation; [a]early response/late response.

Gene	Cold	Wounding	*B. cinerea*	SA	MeJA
SlSR1	−6.8	−3.1	−1.9	+13.4	+6.1
SlSR1L	−2.1	−6.1	−2.5	+5.8	+6.5
SlSR2	>+90.0	>+8.0	>+16.0	>+100.0	>+100.0
SlSR2L	+1.3	−4.2/+2.7[a]	−4.5/+3.4[a]	+2.6	+4.3
SlSR3	−1.8	+1.3/−3.4[a]	+1.3/−3.7[a]	+2.2	+3.8
SlSR3L	+1.5	+9.4	+10.0	+6.3	−2.8/+2.8[a]
SlSR4	−4.0	1.6	+11.8	+2.6	+2.3

factors. First identified in plants (Yang and Poovaiah 2000), *SR/CAMTA*s are present in all plant and animal species surveyed to date. In the vegetative tissues of *Arabidopsis*, *SR/CAMTAs* show differential responses to a variety of environmental signals, including cold, heat shock, wounding, ethylene, SA and MeJA. Knockout of *AtSR1* led to increased accumulation of SA and enhanced disease resistance (Galon et al. 2008, Du et al. 2009), and decreased JA level and reduced wounding and herbivory tolerance (Laluk et al. 2012, Qiu et al. 2012). *AtSR1/CAMTA3* and *AtSR2/CAMTA1* are also important for plant tolerance to low temperature (Doherty et al. 2009). Previously we have shown that *SlSRs* are developmentally regulated and responsive to ethylene treatment in tomato fruit. This study showed that *SlSRs* in fruit have distinct expression patterns to different abiotic and biotic stresses as well as two important stress-related plant hormones, SA and JA, thus are likely to have distinct roles in responses to specific stress signals. Taken together, our results indicate that *SlSRs* could orchestrate the interplay of calcium-mediated signaling with multiple stress signal transduction pathways in fruit tissues.

It has been documented that SR/CAMTAs selectively bind a CGCG-containing DNA sequence. In *Arabidopsis*, AtSR1/CAMTA3 was reported to bind to the DNA motif (G/A/C)CGCG(T/G/C) in vitro (Yang and Poovaiah 2002). In planta, the CGCG box in the promoter region of *EDS1* is confirmed the direct target of AtSR1 (Du et al. 2009). *EDS1* is a key gene for SA biosynthesis. Based on microarray assays, other possible targets identified for AtSR1 include genes encoding expansin, β-1,3-glucanase, phospholipase A2, accelerated cell death protein 6, and senescence associated protein 21 (Galon et al. 2008). Interestingly, the *Drosophila* DmCAMTA also binds to DNA sequences containing the CGCG-core motif, suggesting that CGCG motif is a consensus DNA-binding motif for SR/CAMTAs

(Han et al. 2006, Gong et al. 2007). Therefore, it is reasonable to predict that tomato *SlSRs* also regulate gene expression via binding to CGCG-box *cis*-elements of target genes. Although the tomato genome has been sequenced (Sato et al. 2012), the tools for genome-wide analysis of the *cis*-elements of all tomato genes are still lacking. Therefore, we analyzed the promoter regions of selected tomato orthologs of *Arabidopsis SR/CAMTAs* targets, and found that there exists at least one CGCG box within 2 kb upstream of many of those genes. For example, the tomato *EDS1* (Solyc06g071280) promoter contains a CGCG box located at approximately −1.3 kb. It also showed high expression at the mature green stage (data not shown), suggesting that *SlSRs* regulate SA levels during fruit development and ripening. Further analysis and identification of *SlSRs* target genes is critical to define their functions during fruit ripening and in response to postharvest abiotic and biotic stresses.

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